FEBS Letters 585 (2011) 199–206

journal homepage: www.FEBSLetters.org

Botulinum neurotoxin subtype A2 enters neuronal cells faster than subtype A1

Christina L. Pier [a], Chen Chen [b], William H. Tepp [a], Guangyun Lin [a], Kim D. Janda [c,d], Joseph T. Barbieri [b], Sabine Pellett [a], Eric A. Johnson [a,*]

[a] Department of Bacteriology, University of Wisconsin, Madison, Madison, WI 53706, USA
[b] Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, USA
[c] Department of Chemistry, The Skaggs Institute for Chemical Biology, and Worm Institute for Research and Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[d] Department of Immunology, The Skaggs Institute for Chemical Biology, and Worm Institute for Research and Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

ARTICLE INFO

Article history:
Received 21 September 2010
Revised 23 November 2010
Accepted 25 November 2010
Available online 30 November 2010

Edited by Maurice Montal

Keywords:
Botulinum neurotoxin
BoNT/A1
BoNT/A2
Cell based assay
Cell entry
Neuron

ABSTRACT

Botulinum neurotoxins (BoNTs), the causative agent of human botulism, are the most potent naturally occurring toxins known. BoNT/A1, the most studied BoNT, is also used as an important biopharmaceutical. In this study, the biological activity of BoNT/A1 is compared to that of BoNT/A2 using neuronal cell models. The data obtained indicate faster and increased intoxication of neuronal cells by BoNT/A2 than BoNT/A1, and that the mechanism underlying this increased toxicity is faster and more efficient cell entry that is independent of ganglioside binding. These results have important implications for the development of new BoNT based therapeutics and BoNT countermeasures.

Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

1. Introduction

Botulinum neurotoxins (BoNTs), the most potent protein toxins known, cause botulism and are produced by a heterogenous group of neurotoxigenic bacteria including *Clostridium botulinum*, and select strains of *Clostridium butyricum* and *Clostridium baratii* [1,2]. Based on their antigenic specificity, BoNTs are classified into seven serotypes (A–G) [2], with BoNT/A, B, E, and F accounting for most recorded cases of human botulism [1]. BoNTs are important disease causing agents [1,3], potential bioterrorism agents [4], and pharmaceuticals for treating neuromuscular disorders and cosmetic use [5,6].

BoNTs are 150 kDa proteins consisting of a heavy chain (HC, ~100 kDa) and a light chain (LC, ~50 kDa) linked by a disulfide bond. BoNT intoxication of neuronal cells requires multiple steps (reviewed in [7]). The C-terminal domain of the HC (HCR/A) binds dual receptors, first a ganglioside on the cell surface and then a protein receptor upon neurotransmitter vesicle fusion with the plasma membrane [8]. The toxin–receptor complex then enters the neuron via endocytosis (reviewed in [9–11]), and acidification within the endosome leads to a conformational change and formation of a HC channel in the endocytic vesicle membrane [12,13]. The translocation domain of the HC then conducts the LC through the HC-channel into the cytosol and the disulfide bond linking the HC and LC is reduced. The released LC refolds to a catalytically competent conformation within the cytosol [12,13]. LCs are zinc endopeptidases that cleave and inactivate Soluble N-ethylmaleimide Sensitive Fusion Attachment Protein Receptors (SNARE), which are essential for neurotransmitter release [14–19].

Several subtypes of most BoNT serotypes have been identified by nucleotide sequence analysis (reviewed in [20]). BoNT/A has been divided into five subtypes (A1–A5) [21–28], but limited information is available on biological and functional characteristics among BoNT subtypes. Prior analyses of BoNT/A subtypes include studies using recombinant LC endopeptidases of BoNT/A1–A4 expressed in *Escherichia coli*, which indicate that all four subtypes bind SNAP-25 with similar affinity, but that rLC/A3 and rLC/A4 cleave SNAP-25 significantly less efficiently than rLC/A1 and rLC/A2 [29]. Sequence and structural analyses show that BoNT/A1 and A2 differ by ~10% at the amino acid level, with the greatest difference in the receptor binding domain. In

* Corresponding author. Fax: +1 608 263 1114.
E-mail address: eajohnso@wisc.edu (E.A. Johnson).

0014-5793/$36.00 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2010.11.045

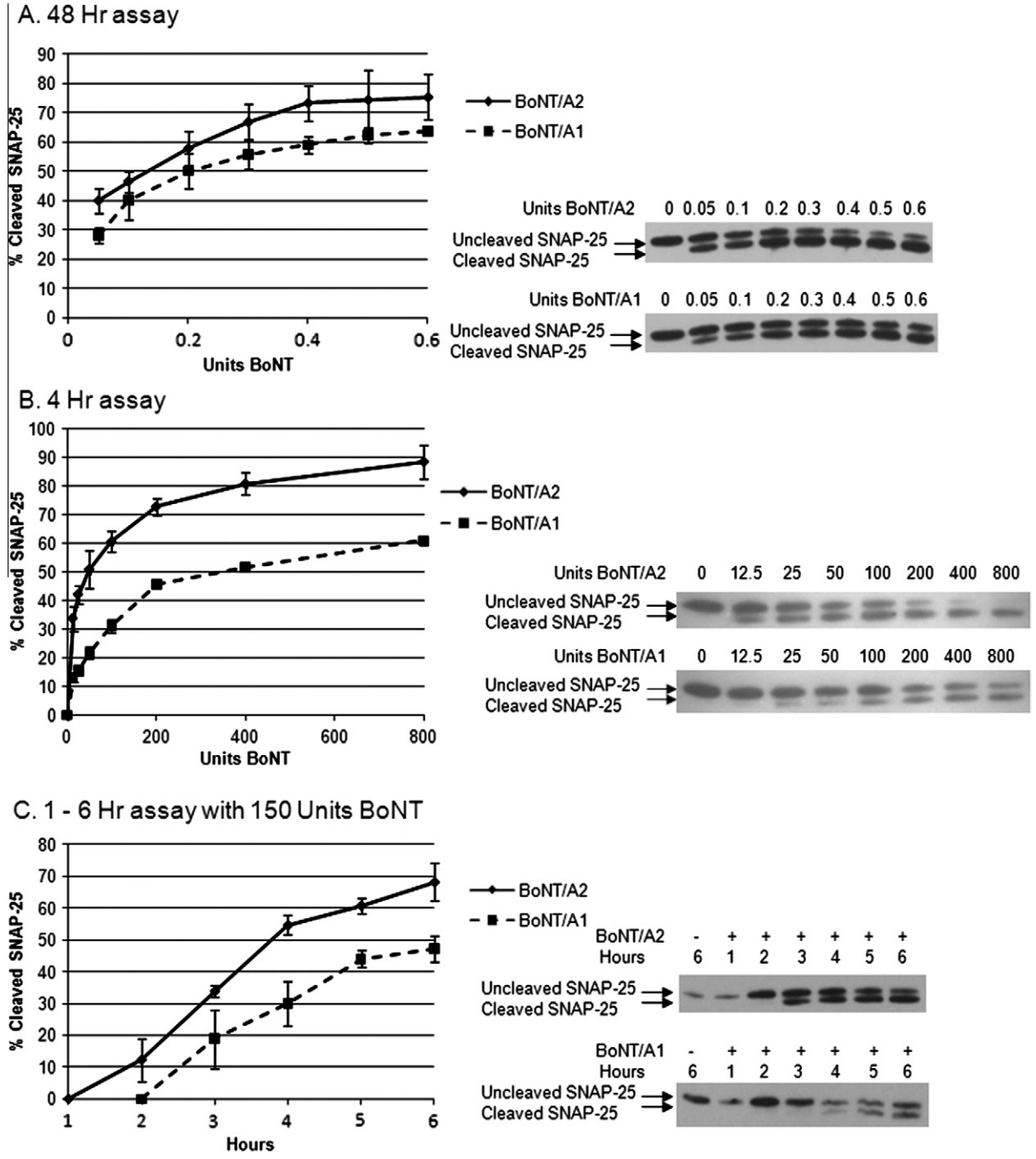

Fig. 1. Sensitivity of RSC cells to BoNT/A2 and A1. (A) 48 h exposure; (B) 4 h exposure; (C) exposure to 150 Units for 1–6 h. Representative Western blots are shown.

contrast, the ganglioside binding pocket and the catalytic LC domain are highly conserved [21,25]. BoNT/A1 and A2 have significantly altered monoclonal antibody binding and inhibition profiles by monoclonal antibodies [25], but no differences were observed in protection against the two subtypes by polyclonal antisera raised against BoNT/A1 [30].

Of the five BoNT/A subtypes identified, BoNT/A1, A2, and A5 have now been purified [31,32], enabling detailed studies which are needed to facilitate the increasing use of BoNTs for medicinal use and to develop effective countermeasures to intoxication by the various subtypes of BoNTs. A recent report suggests that BoNT/A2 is more potent than BoNT/A1 in vivo [33]. The data presented here demonstrate that BoNT/A2 is more potent than BoNT/A1 in neuronal cells due to faster cell entry.

2. Materials and methods

2.1. Botulinum neurotoxins

BoNT/A1, A2, and E were purified as previously described [34,32,35]. The specific activities were determined via the mouse bioassay [36,37] to be 1.3×10^8 LD_{50} Units/mg for BoNT/A1, 4.3×10^8 Units/mg for BoNT/A2, and 0.76×10^8 Units/mg for BoNT/E.

2.2. Reagents

Tissue culture reagents, media, and Western blot supplies were obtained from Invitrogen (Carlsbad, CA). Neuro-2a cells were pur-

chased from American Type Culture Collection and rat E18-cortical neurons from BrainBits, LLC. CITIFLUOR AF-3 was purchased from Electron Microscopy Sciences and toosendanin was purchased from AvaChem Scientific (San Antonio, TX) with purity confirmed as described [38]. All other chemicals were obtained from SIGMA.

2.3. Primary rat spinal cord (RSC) cells assay

The preparation of spinal cord cells and toxin assays were previously described [39,40]. The cells were plated and maintained in culture medium (CM, Neurobasal medium supplemented with B27, glutamax, and penicillin/streptomycin). Cells were matured for $\geqslant$18 days prior to use. BoNT was added in 50 μl CM per well and incubated for the indicated times at 37 °C in a humidified 5% CO_2 atmosphere. The cells were lysed in 75 μl of LDS lysis buffer and analyzed by Western blot using a monoclonal anti-SNAP-25 antibody (Synaptic Systems) and densitometry as described previously [39]. All assays were performed in triplicate.

For the toosendanin inhibition assay, 500 Units of BoNT were added to pre-warmed stimulation medium (SM: neurobasal medium supplemented with 56 mM KCl and 2.2 mM $CaCl_2$ (Invitrogen custom media)) in a total volume of 50 μl per well and incubated at 37 °C for 6 min. Cells were washed twice with 100 μl CM followed by incubation at 37 °C, 5% CO_2 for 24 h. Toosendanin (1 μM) [38] was added in CM, 1% DMSO at the indicated times after toxin exposure. All assays were performed in triplicate.

To study the duration of action of BoNT/A1 and A2 in neurons, RSC cells (400 000 per well) were plated into collagen coated 24-well plates (BD Biosciences). Five Units BoNT/A1 or A2 was added in 300 μl CM per well and incubated for 48 h at 37 °C, 5% CO_2. The cells were washed 3× with 500 μl CM and incubated at 37 °C, 5% CO_2 for 22 weeks with bi-weekly media changes. Cells were harvested at the indicated times and analyzed by Western blot as described above. All time points were tested in at least duplicate.

2.4. Neuro-2a cells BoNT assay

Neuro-2a cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with non-essential amino acids, 10% fetal bovine serum, and penicillin/streptomycin. The cells were seeded into 96-well TPP dishes (MidSci) to ~50% confluence and exposed the following day to the indicated amounts of BoNT/A1 or BoNT/A2 in 50 μl DMEM for 24 h or 48 h. Cells lysates were analyzed by Western blot as described above. To pre-load cells with gangliosides, the cells were seeded into a 96-well collagen coated plate and after 24 h the medium was changed to CM (serum free) containing 25 μg/ml of GT1b (Sigma). After 24 h, the cells were exposed to BoNT/A1 or BoNT/A2 in 50 μl CM for 24 h or 48 h as indicated and cell lysates were analyzed as above. All assays were performed in triplicate.

2.5. HCR/A1 and HCR/A2 interactions with rat cortical neurons

Synthetic BoNT/A1, BoNT/A2, and TeNT HC receptor binding domains (HCR) containing a 3xFlag epitope were expressed and purified as described previously [41]. HCR/A1 and A2 consist of residues 870–1296 of each BoNT/A subtype, while HCR/T consists of residues 865–1315 of TeNT. Rat E18-cortical neurons were cultured on poly-D-lysine-coated glass cover slips in Neurobasal Medium with 2 mM glutamine and B27 supplement, for 10–14 days prior to use.

Binding of HCRs to neurons was examined at 4 °C for 60 min with 40 nM HCR/A1, HCR/A2, or HCR/T in low potassium (15 mM HEPES, 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl, 0.5 mM MgCl, pH 7.4) or high potassium (15 mM HEPES, 95 mM NaCl, 56 mM KCl, 2.2 mM CaCl, 0.5 mM MgCl, pH 7.4) buffer. Entry and trafficking of HCRs in neurons was measured at 37 °C for 5 min in low or high potassium buffer. Cells were washed with Dulbeccos phosphate buffered saline (DPBS) and fixed with 4% (w/v) paraformaldehyde in DPBS. Cells were incubated with 150 mM glycine in DPBS, washed with DPBS and blocked with blocking solution [10% (v/v) normal goat serum, 2.5% (w/v) cold fish skin gelatin (Sigma), 0.1% Triton-X-100, and 0.05% Tween-20 in DPBS] for 1 h at RT, followed by incubation with mouse anti-FLAG M2 antibody (SIGMA, 1:15 000 dilution) in antibody solution [5% (v/v) normal goat serum, 1% (w/v) cold fish skin gelatin, 0.1% Triton-X-100, 0.05% Tween-20 in DPBS] overnight at 4 °C. Cells were washed with DPBS and incubated with goat anti-mouse IgG Alexa568 (Molecular Probes; 1:250 dilution) and alexa 488-cholera toxin B subunit (CTB) as a surface marker. Co-localization experiments were performed using rabbit anti-Rab5 antibody (Abcam, 1:1000 dilution) and guinea pig anti-synaptophysin 1 (Synaptic Systems, 1:2000 dilution) followed by goat anti-mouse IgG Alexa-568, goat anti-rabbit IgG Alexa-488 and goat anti-guinea pig IgG Alexa-633 (1:250 dilution), respectively. Mounting reagent CITIFLUOR AF-3 was added to the well and average fluorescence and standard deviation from eight random fields was determined. Images were captured with a Nikon TE2000 TIRF microscope equipped with a CFI Plan Apo VC 100X Oil, NA 1.4 type objective. Image analyses were performed using MetaMorph version 7.0 and figures were compiled using Photoshop CS (Adobe). HCR binding to cultured cells was quantified by two-tailed Student's *t*-test and *P* values were calculated using GraphPad Prism 5 software.

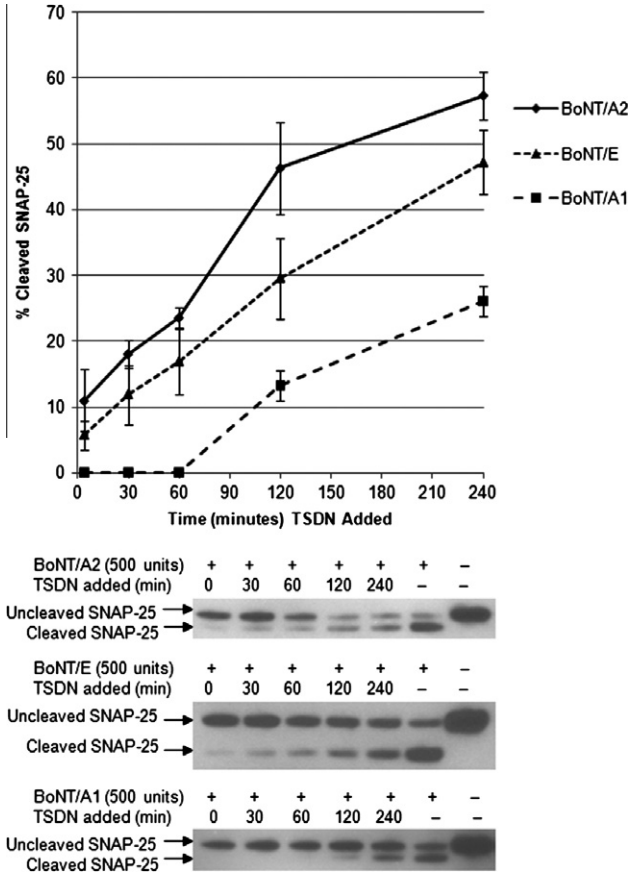

Fig. 2. Cell entry kinetics of BoNT/A2, E, and A1 in RSC cells. RSC cells were exposed to 500 U BoNT for 6 min, and toosendanin was added as indicated. Representative Western blots are shown.

3. Results

3.1. BoNT/A2 is more potent in neuronal cells than BoNT/A1

The sensitivity of RSC cells to BoNT/A1 has previously been established [39,40]. Sensitivity of RSC cells to BoNT/A1 and A2 was compared by exposing matured RSC cells to serial dilutions of BoNT/A1 and A2 for 48 h and 4 h. The sensitivity was consistently higher for BoNT/A2 than A1, with 50% SNAP-25 cleavage achieved at 0.193 Units of A1 and 0.133 Units of A2 after 48 h exposure (Fig. 1A) and at 230 Units of A1 and 70 Units of A2 after 4 h exposure (Fig. 1B). These data led to the hypothesis that uptake of BoNT/A2 into RSC cells may be faster than uptake of BoNT/A1.

3.2. BoNT/A2 enters neuronal cells faster than BoNT/A1

To examine this further, RSC cells were exposed to 150 Units of each BoNT subtype for 1–6 h and cell lysates were assayed for SNAP-25 cleavage. SNAP-25 cleavage was observed 1 h earlier and progressed more rapidly for BoNT/A2 than BoNT/A1 (Fig. 1C).

Toosendanin, a specific inhibitor that blocks translocation of the BoNT LC into the cytosol [38], was utilized to further analyze the entry kinetics of BoNT/A1 and A2 into RSC cells. BoNT/E was used as a control for this assay, because it enters cells significantly faster than BoNT/A1 [42]. To achieve uniform endocytic uptake of the BoNT subtypes, RSC cells were briefly (6 min) exposed to toxin during chemical depolarization, followed by 24 h incubation [42]. Toosendanin was added in 30 min intervals. SNAP-25 cleavage was first observed when toosendanin was added 150 min after BoNT/A1 exposure, whereas the same amount of cleavage was evident as early as 30 min after BoNT/A2 and BoNT/E exposure (Fig. 2). This indicates that BoNT/A2 enters cells more rapidly than BoNT/A1.

3.3. Greater cellular toxicity by BoNT/A2 is independent of ganglioside binding

BoNT/A1 and A2 activity were compared further in the more defined background of the Neuro-2a cell line. Neuro-2a cells express only the C subtype of the SV2 protein receptor [43] and BoNT/A1 sensitivity is increased dramatically if the cells are pre-loaded with GT1b gangliosides [44]. The sensitivity of this cell line to BoNT/A1 and A2 was examined after 24 and 48 h exposure with and without GT1b pre-loading (Fig. 3). BoNT/A2 was about 125-fold (24 h) and 25-fold (48 h) more potent than BoNT/A1 in inducing SNAP-25 cleavage (Fig. 3). When the Neuro-2a cells were pre-loaded with GT1b, sensitivity to both subtypes was increased dramatically and similarly. This confirms the increased neurotoxicity of BoNT/A2 in neuronal cells and indicates that ganglioside binding is not responsible for that difference.

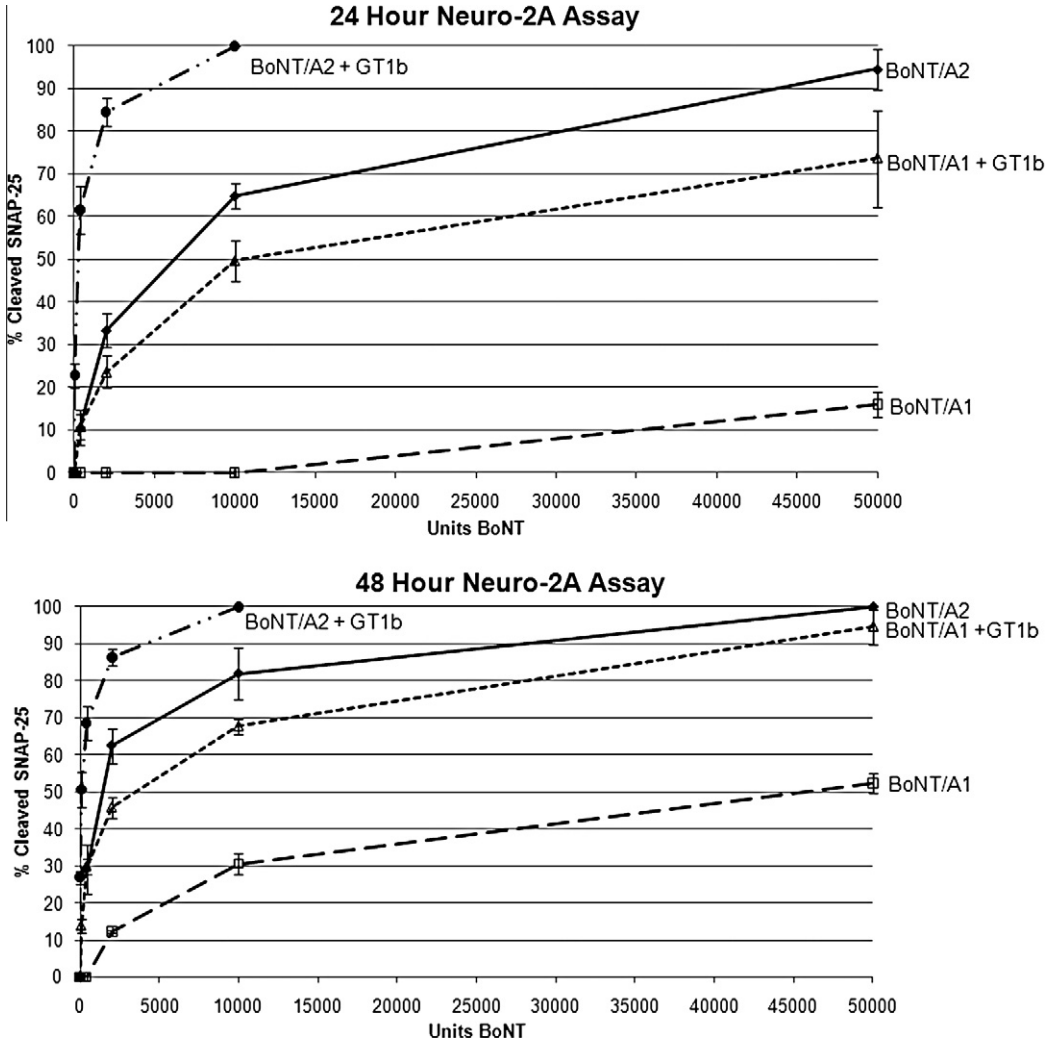

Fig. 3. Sensitivity of Neuro-2a cells to BoNT/A2 and A1. Cells were exposed to BoNT for 24 h or 48 h with or without GT1b pre-loading.

3.4. BoNT/A1 and BoNT/A2 have a long duration of activity in neuronal cells

Duration of BoNT activity in neuronal cells was examined as has been previously described [42]. RSC cells were exposed to 5 Units of BoNT/A1 or BoNT/A2 for 48 h followed by removal of all extracellular toxin and incubation for ⩽22 weeks. SNAP-25 cleavage in cell lysates was assessed every 2–4 weeks. Over 22 weeks of analysis, SNAP-25 cleavage remained constant at ~70%, indicating no recovery of the cells. Similar exposure of cells with BoNT/E resulted in complete recovery (detection of only full-length SNAP-25) within 2 weeks, as has been previously reported [42].

3.5. HCR/A2 enters neurons more efficiently than HCR/A1

To further address the mechanism responsible for faster cell entry by BoNT/A2, rat cortical neurons were exposed to recombinant HCR derivatives (HCR/A1, HCR/A2, and HCR/T) at 4 °C to measure intrinsic plasma membrane binding HCR/A1 and HCR/A2 showed no detectable binding to neurons (Fig. 4) under conditions where HCR/T (HCR of tetanus toxin) binding was observed, indicating

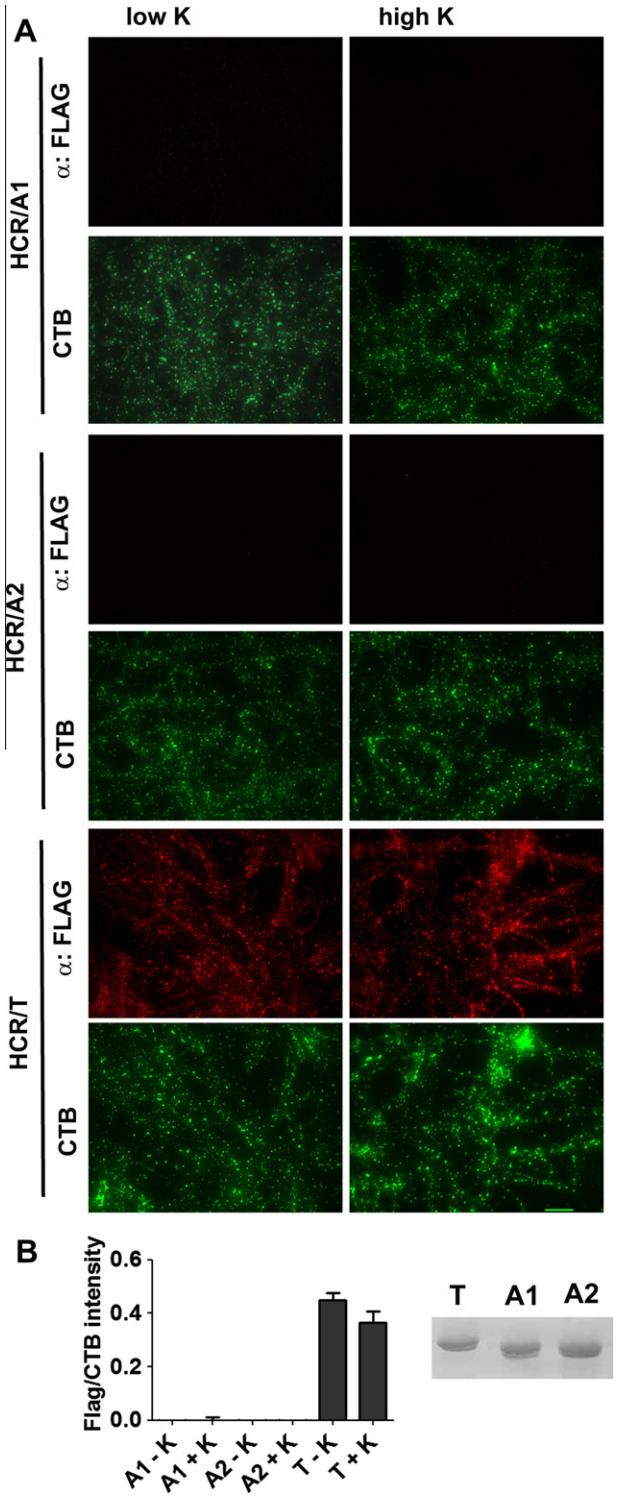

Fig. 4. HCR/A1, HCR/A2, and HCR/T binding to neurons. Rat cortical neurons were incubated with HCRs in low or high potassium buffer at 4 °C. Bound HCR was detected using mouse-a-FLAG IgG. CTB was used as a surface marker. (A) Representative microscopic image. (B) Quantified HCRs bound determined as the ratio of anti-FLAG antibody/CTB staining and coomassie stained gel.

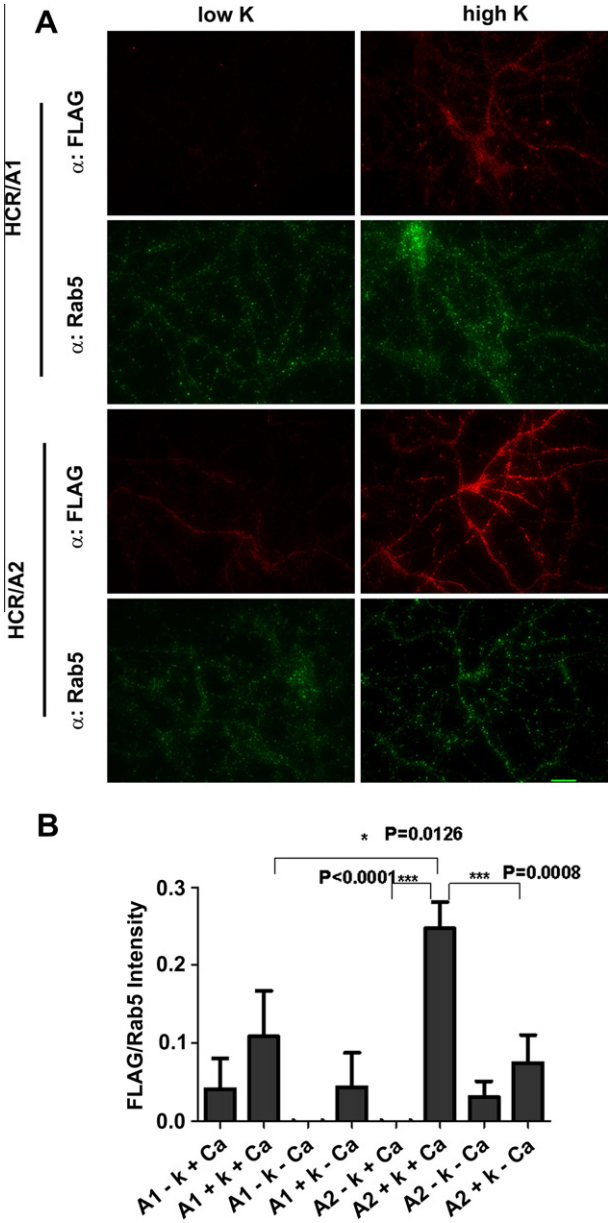

Fig. 5. HCR/A1 and HCR/A2 entry to neurons. HCR/A1 or HCR/A2 were incubated with rat cortical neurons in low or high potassium buffer for 5 min at 37 °C. Bound HCR (anti-FLAG) and marker proteins (anti-Rab) were detected by immuno fluorescence. (A) A representative field is shown. (B) Fluorescence intensity of the ratio of anti-FLAG (HCR)/anti-Rab5 as a marker for HCR binding and internalization.

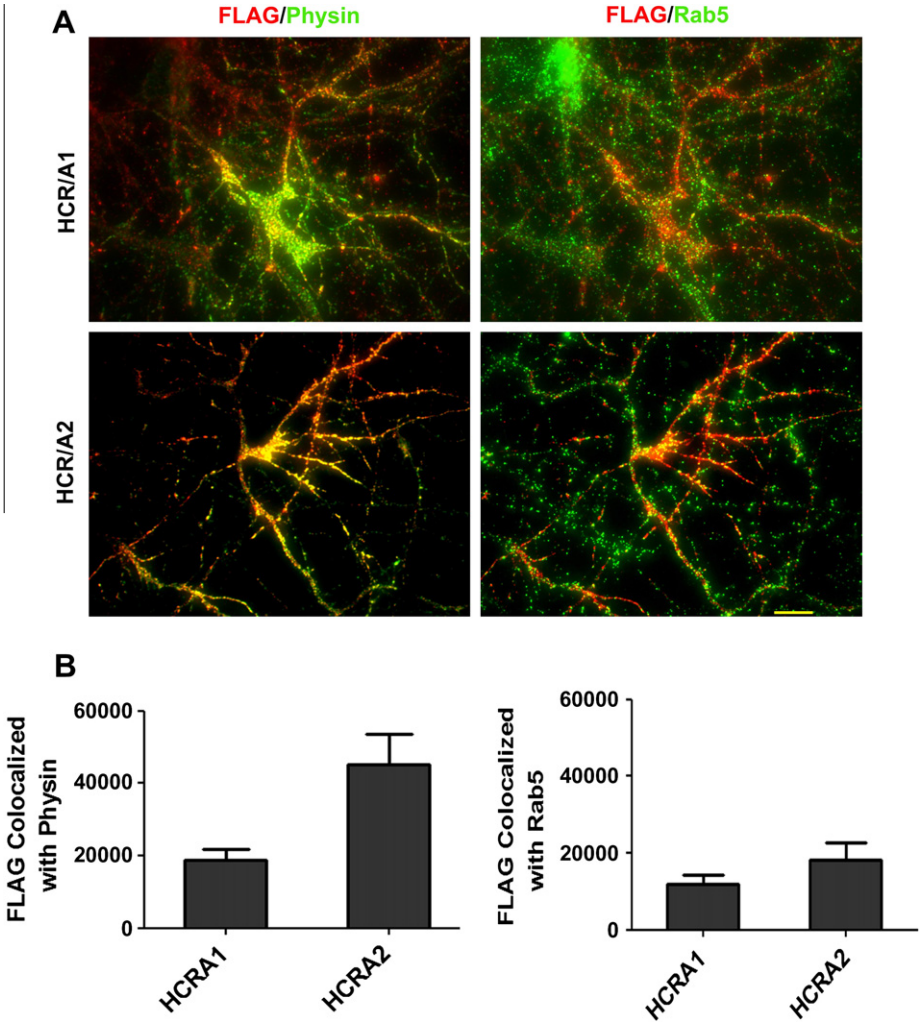

Fig. 6. HCR/A1 and HCR/A2 co-localization with synaptophysin 1 (Physin) and Rab5. HCR/A1 and A2 were incubated with rat cortical neurons in high potassium buffer for 5 min at 37 °C. (A) HCR co-localization (FLAG epitope) with synaptophysin 1 and Rab5. (B) Co-localization of HCR/A1 A2 (red) with Rab5 and synaptophysin 1 (green) was plotted in fluorescence intensities units (arbitrary).

low intrinsic binding potential of BoNT/A1 and A2 to the plasma membrane of neurons. Cell entry and trafficking were measured by exposure of neurons to HCR/A1 and A2 at 37 °C in low and high potassium buffer to mimic resting and activated neurons, respectively. In low potassium, HCR/A1 and HCR/A2 entry into neurons was low (Fig. 5) consistent with the need for synaptic vesicle fusion to expose SV2, the BoNT/A protein receptor, on the plasma membrane. In high potassium, HCR/A1 and HCR/A2 entry were detected with significantly greater amounts of HCR/A2 cell associated relative to HCR/A1.

To further characterized the pathway that HCR/A utilizes to enter neurons, co-localization of the HCRs with synaptic vesicle (synaptophysin 1)- and endocytosis (Rab5)-marker proteins was measured. In high potassium buffer, HCR/A1 and A2 co-localized to both marker proteins. Both demonstrate greater co-localization to the synaptophysin 1 than to Rab5, which is consistent with cell entry via synaptic vesicle endocytosis (Fig. 6). HCR/A2 showed a statistically greater amount of co-localization with synaptic vesicles than HCR/A1, indicating that HCR/A2 entered neurons more efficiently than HCR/A1.

4. Discussion

In this study, the biological activity of BoNT/A2 is compared to BoNT/A1 using neuronal cell models. Exposure of RSC cells and Neuro-2a cells to BoNT/A1 or A2 showed increased sensitivity of the cells to BoNT/A2 (Figs. 1 and 3), suggesting that the biological potency of BoNT/A2 is greater than BoNT/A1 in neuronal cells. Preloading Neuro-2a cells with GT1b gangliosides resulted in equally increased sensitivity to both BoNT/A1 and A2, indicating that the greater intoxication by BoNT/A2 is independent of ganglioside binding, which correlates with the high degree of sequence similarity in the gangliosides binding pocket of BoNT/A1 and A2 [21,25].

Studies with toosendanin, a specific inhibitor of BoNT LC translocation [38], indicated that the enhanced neurotoxicity of BoNT/A2 compared to A1 is due to more rapid translocation of BoNT/A2 or to a step in the intoxication process preceding translocation (Fig. 2). A mechanism utilizing faster cell entry rather than increased LC activity is consistent with earlier studies showing that rLCs of BoNT/A1 and A2 had similar catalytic potentials, a high degree of sequence similarity in the catalytic LC domain and the ganglioside binding pocket, and the greatest difference in amino acid sequence in the protein receptor binding domain [21,25]. BoNT/A2's fast cellular entry mandates that for toosendanin to be fully effective it needs to be used in a prophylactic fashion, whereas BoNT/A1's slower cellular entry provides a window of opportunity for toosendanin to be effective both as a prophylactic and a therapeutic. The lack of any SNAP-25 recovery in RSC cells incubated for up to 22 weeks after BoNT/A1 and A2 exposure indicates that both

toxin subtypes have a duration of action of at least 22 weeks in cultured neurons.

The mechanism of faster cell entry by BoNT/A2 was further examined using recombinant heavy chain receptor domains (HCR) to measure cellular entry and trafficking into primary cortical neurons, a neuronal cell model previously established for similar studies [45]. The greater amount of cell associated HCR/A2 at 37 °C, relative to HCR/A1, indicated that HCR/A2 possessed a greater capacity to enter neurons. The unique entry properties of the BoNT/A subtypes could be explained by differential affinity for SV2 or a specific SV2 isotype. Preferential binding of BoNT/A1 to a SV2-isotypes has previously been observed [46,47]. Other steps that may account for the differential entry properties of the BoNT/A subtypes are the rate of entry into synaptic vesicle derived endosomes or the rate of translocation of the LC into the cell cytosol.

Taken together, the data presented in this study confirm faster and increased intoxication of neuronal cells by BoNT/A2 compared to A1 due to faster cell entry by BoNT/A2, while maintaining similar longevity of action. These data are consistent with a recent report suggesting greater potency of BoNT/A2 than A1 in vivo [33]. Future experiments resolving the precise mechanism responsible for the different entry properties of these BoNT/A subtypes will provide insight into the mechanism of BoNT/A entry into neurons and provide information to manipulate the entry properties of the BoNTs to facilitate their utilization for intervention of neurological injury, inhibitor design, and enhanced activity as a pharmaceutical [3,5,48].

Acknowledgements

The project described was supported by the University of Wisconsin, Madison, as well as by the National Institute of Allergy and Infectious Diseases (Award Number R21AI082826), and by the NIH/NIAID Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (RCE) Program. The Johnson Lab wishes to acknowledge membership within and support from the Pacific Southwest Regional Center of Excellence grant U54 AI065359. The Barbieri and Johnson Labs acknowledge membership and support from the Great Lakes Regional Center of Excellence U54 AI057153. K.D. Janda acknowledges support from the Skaggs Institute for Chemical Biology as well as the NIAID AI080671.

References

[1] Johnson, E. (2005) *Clostridium botulinum* and *Clostridium tetani*. Topley and Wilson's Microbiol. Microb. Infect. 8, 1035–1088.
[2] Gimenez, D.F. and Gimenez, J.A. (1995) The typing of botulinal neurotoxins. Int. J. Food Microbiol. 27, 1–9.
[3] Montecucco, C. and Molgo, J. (2005) Botulinal neurotoxins: revival of an old killer. Curr. Opin. Pharmacol. 5, 274–279.
[4] Arnon, S.S., Schechter, R., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Hauer, J., Layton, M., Lillibridge, S., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Swerdlow, D.L. and Tonat, K.Working Group on Civilian Biodefense (2001) Botulinum toxin as a biological weapon: medical and public health management. JAMA 285, 1059–1070.
[5] Foster, K.A., Bigalke, H. and Aoki, K.R. (2006) Botulinum neurotoxin – from laboratory to bedside. Neurotoxicol. Res. 9, 133–140.
[6] Chaddock, J.A. and Marks, P.M. (2006) Clostridial neurotoxins: structure–function led design of new therapeutics. Cell. Mol. Life Sci. 63, 540–551.
[7] Montal, M. (2010) Botulinum neurotoxin: a marvel of protein design. Annu. Rev. Biochem..
[8] Montecucco, C. (1986) How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem. Sci. 11, 314–317.
[9] Montecucco, C., Rossetto, O. and Schiavo, G. (2004) Presynaptic receptor arrays for clostridial neurotoxins. Trends Microbiol. 12, 442–446.
[10] Schiavo, G., Matteoli, M. and Montecucco, C. (2000) Neurotoxins affecting neuroexocytosis. Physiol. Rev. 80, 717–766.
[11] Simpson, L.L. (2004) Identification of the major steps in botulinum toxin action. Annu. Rev. Pharmacol. Toxicol. 44, 167–193.
[12] Fischer, A. and Montal, M. (2007) Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. J. Biol. Chem. 282, 29604–29611.
[13] Fischer, A. and Montal, M. (2007) Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. Proc. Natl. Acad. Sci. USA 104, 10447–10452.
[14] Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T.C., Niemann, H. and Jahn, R. (1993) Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature 365, 160–163.
[15] Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T.C., Jahn, R. and Niemann, H. (1994) Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J. Biol. Chem. 269, 1617–1620.
[16] Schiavo, G., Shone, C.C., Rossetto, O., Alexander, F.C. and Montecucco, C. (1993) Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol. Chem. 268, 11516–11519.
[17] Schiavo, G., Rossetto, O., Tonello, F. and Montecucco, C. (1995) Intracellular targets and metalloprotease activity of tetanus and botulism neurotoxins. Curr. Top. Microbiol. Immunol. 195, 257–274.
[18] Montecucco, C. and Schiavo, G. (1994) Mechanism of action of tetanus and botulinum neurotoxins. Mol. Microbiol. 13, 1–8.
[19] Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E.M., Sudhof, T.C. and Jahn, R. (1994) Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J. Biol. Chem. 269, 12764–12772.
[20] Peck, M.W. (2009) Biology and genomic analysis of *Clostridium botulinum*. Adv. Microb. Physiol. 55, 183. 265, 320.
[21] Arndt, J.W., Jacobson, M.J., Abola, E.E., Forsyth, C.M., Tepp, W.H., Marks, J.D., Johnson, E.A. and Stevens, R.C. (2006) A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1–A4. J. Mol. Biol. 362, 733–742.
[22] Dover, N., Barash, J.R. and Arnon, S.S. (2009) Novel *Clostridium botulinum* toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. J. Clin. Microbiol. 47, 2349–2350.
[23] Carter, A.T., Paul, C.J., Mason, D.R., Twine, S.M., Alston, M.J., Logan, S.M., Austin, J.W. and Peck, M.W. (2009) Independent evolution of neurotoxin and flagellar genetic loci in proteolytic *Clostridium botulinum*. BMC Genom. 10, 115.
[24] Hill, K.K., Smith, T.J., Helma, C.H., Ticknor, L.O., Foley, B.T., Svensson, R.T., Brown, J.L., Johnson, E.A., Smith, L.A., Okinaka, R.T., Jackson, P.J. and Marks, J.D. (2007) Genetic diversity among Botulinum neurotoxin-producing clostridial strains. J. Bacteriol. 189, 818–832.
[25] Smith, T.J., Lou, J., Geren, I.N., Forsyth, C.M., Tsai, R., Laporte, S.L., Tepp, W.H., Bradshaw, M., Johnson, E.A., Smith, L.A. and Marks, J.D. (2005) Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. Infect. Immun. 73, 5450–5457.
[26] Gimenez, D.F. (1984) *Clostridium botulinum* subtype Ba. Zentralbl. Bakteriol. Mikrobiol. Hyg. A 257, 68–72.
[27] Kozaki, S., Nakaue, S. and Kamata, Y. (1995) Immunological characterization of the neurotoxin produced by *Clostridium botulinum* type A associated with infant botulism in Japan. Microbiol. Immunol. 39, 767–774.
[28] Sakaguchi, G., Sakaguchi, S., Kamata, Y., Tabita, K., Asao, T. and Kozaki, S. (1990) Distinct characters of *Clostridium botulinum* type A strains and their toxin associated with infant botulism in Japan. Int. J. Food Microbiol. 11, 231–241.
[29] Henkel, J.S., Jacobson, M., Tepp, W., Pier, C., Johnson, E.A. and Barbieri, J.T. (2009) Catalytic properties of botulinum neurotoxin subtypes A3 and A4 (dagger). Biochemistry 48, 2522–2528.
[30] Baldwin, M.R., Tepp, W.H., Pier, C.L., Bradshaw, M., Ho, M., Wilson, B.A., Fritz, R.B., Johnson, E.A. and Barbieri, J.T. (2005) Characterization of the antibody response to the receptor binding domain of botulinum neurotoxin serotypes A and E. Infect. Immun. 73, 6998–7005.
[31] DasGupta, B.R. and Sathyamoorthy, V. (1984) Purification and amino acid composition of type A botulinum neurotoxin. Toxicon 22, 415–424.
[32] Lin, G., Tepp, W.H., Pier, C.L., Jacobson, M.J. and Johnson, E.A. (2010) Expression of the *Clostridium botulinum* A2 neurotoxin gene cluster proteins and characterization of the A2 complex. Appl. Environ. Microbiol. 76, 40–47.
[33] Torii, Y., Kiyota, N., Sugimoto, N., Mori, Y., Goto, Y., Harakawa, T., Nakahira, S., Kaji, R., Kozaki, S. and Ginnaga, A. (2010) Comparison of effects of botulinum toxin subtype A1 and A2 using twitch tension assay and rat grip strength test. Toxicon.
[34] Malizio, C.J., Goodnough, M.C. and Johnson, E.A. (2000) Purification of *Clostridium botulinum* type A neurotoxin. Methods Mol. Biol. 145, 27–39.
[35] Prabakaran, S., Tepp, W. and DasGupta, B.R. (2001) Botulinum neurotoxin types B and E: purification, limited proteolysis by endoproteinase Glu-C and pepsin, and comparison of their identified cleaved sites relative to the three-dimensional structure of type A neurotoxin. Toxicon 39, 1515–1531.
[36] Hatheway, C.L. (1988) Botulism in: Laboratory Diagnosis of Infectious Diseases: Principles and Practice (Balows, A., Hausler, W.J. Jr.Jr., Ohashi, M. and Turano, A.J., Eds.), pp. 111–133, Springer-Verlag, London.
[37] Schantz, E.J. and Kautter, D.A. (1978) Standardized assay for *Clostridium botulinum* toxins. J. Assoc. Off. Anal. Chem. 61, 96–99.
[38] Fischer, A., Nakai, Y., Eubanks, L.M., Clancy, C.M., Tepp, W.H., Pellett, S., Dickerson, T.J., Johnson, E.A., Janda, K.D. and Montal, M. (2009) Bimodal modulation of the botulinum neurotoxin protein-conducting channel. Proc. Natl. Acad. Sci. USA 106, 1330–1335.

[39] Pellett, S., Tepp, W.H., Clancy, C.M., Borodic, G.E. and Johnson, E.A. (2007) A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. FEBS Lett. 581, 4803–4808.
[40] Pellett, S., Tepp, W.H., Toth, S.I. and Johnson, E.A. (2010) Comparison of the primary rat spinal cord cell (RSC) assay and the mouse bioassay for botulinum neurotoxin type A potency determination. J. Pharmacol. Toxicol. Methods.
[41] Chen, C., Baldwin, M.R. and Barbieri, J.T. (2008) Molecular basis for tetanus toxin coreceptor interactions. Biochemistry 47, 7179–7186.
[42] Keller, J.E., Cai, F. and Neale, E.A. (2004) Uptake of botulinum neurotoxin into cultured neurons. Biochemistry 43, 526–532.
[43] Dong, M., Yeh, F., Tepp, W.H., Dean, C., Johnson, E.A., Janz, R. and Chapman, E.R. (2006) SV2 is the protein receptor for botulinum neurotoxin A. Science 312, 592–596.
[44] Yowler, B.C., Kensinger, R.D. and Schengrund, C.L. (2002) Botulinum neurotoxin A activity is dependent upon the presence of specific gangliosides in neuroblastoma cells expressing synaptotagmin I. J. Biol. Chem. 277, 32815–32819.
[45] Karalewitz, A.P., Kroken, A.R., Fu, Z., Baldwin, M.R., Kim, J.J. and Barbieri, J.T. (2010) Identification of a unique ganglioside binding loop within botulinum neurotoxins C and D-SA. Biochemistry 49, 8117–8126.
[46] Baldwin, M.R. and Barbieri, J.T. (2007) Association of botulinum neurotoxin serotypes A and B with synaptic vesicle protein complexes. Biochemistry 46, 3200–3210.
[47] Poea-Guyon, S., Amar, M., Fossier, P. and Morel, N. (2006) Alternative splicing controls neuronal expression of v-ATPase subunit a1 and sorting to nerve terminals. J. Biol. Chem. 281, 17164–17172.
[48] Truong, D.D., Stenner, A. and Reichel, G. (2009) Current clinical applications of botulinum toxin. Curr. Pharm. Des. 15, 3671–3680.